## UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicants | Rajagopalan et al. |
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| Filing Date | March 24, 2004 |
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| Attorney Docket No. | 073979.30 |

Cincinnati, Ohio                                                                                  March 13, 2008

### DECLARATION OF RAGHAVAN RAJAGOPALAN, Ph.D.
### PURSUANT TO 37 C.F.R.§1.132

I, RAGHAVAN RAJAGOPALAN, declare as follows:

1. I am a named inventor in the above-identified patent application.

2. I hold a Ph.D. in Organic Chemistry from Columbia University. I have 25 years of experience in the synthesis and use of compounds for medical diagnosis and therapy, which is the subject of the application. I have read the November 27, 2007 Office Action and understand the Examiner's position.

3. My claims recite a method for performing a phototherapeutic procedure by administering an effective amount of an organic azide photosensitizer having the formula $E - L - Ar - X - N_3$. Each of E, L, Ar, and X are identified as in originally filed claim 1. In the Election, E = bombesin receptor binding molecule; $L = (CH_2)_a$; a = 1; Ar = benzene; and X = single bond.

4. The Examiner has rejected claims 11, 12, and 21-27 under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. Specifically, the Examiner finds that "one skilled in the art is not taught how or even if the bio-molecule will attach to a $CH_2$ group. Nor is there any indication that this compound would work similar to the disclosed compounds in a method of performing a phototherapeutic procedure."

5. I respectfully disagree. As one example, a person of ordinary skill in the art knows traditional ways for alkylating an aromatic compound, such as benzene. For example, it is common in the art to use the -SH or $-NH_2$ group in a biomolecule, e.g., protein or peptide, to alkylate the photoactive component. This can be accomplished by a halogenating step to produce $Br-CH_2-Ar-$

X-N$_3$, using either molecular bromine or N-bromosuccinimide, and then linking E (through the -SH or -NH$_2$ group) to result in E-CH$_2$-Ar-X-N$_3$ where, as claimed, (CH$_2$)a is linker L between E and Ar. Thus, in my method, Ar is linked by CH$_2$ as linker L. In support, I have attached House, Modern Synthetic Reactions, Second Edition 1972, W.A. Benjamin, Inc. Menlo Park CA, pp. 478-490, and Hermanson, Bioconjugate Techniques, Academic Press New York, pp. 146-148 (1996) that describes these reactions.

6. As another example, a person of ordinary skill in the art knows a reductive amination process, wherein E-NH$_2$ and Ar-CHO react, in the presence of a mild reducing agent, sodium cyanoborohydride, or sodium triacetoxyborohydride, to form E-NH-CH$_2$-Ar-X-N$_3$. In support, I have attached Morrison and Boyd, Organic Chemistry, Allyn and Bacon, Boston, 1969, pp. 733-734.

7. The specification, at p. 12 line 14 to p. 20 line 6, also provides references disclosing how E will attach to a CH$_2$ group, and describes that it will work similar to the disclosed compounds in a method of performing a phototherapeutic procedure.

8. In addition, the specification incorporates by reference U.S. Patent No. 5,714,342, which discloses a peptide (a biological molecule) attached to a CH$_2$ moiety, and the use of this moiety (published application at col. 15 line 60 to col. 34 line 44).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are to be true; and further that these statement were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the subject application or any patent issued thereon.

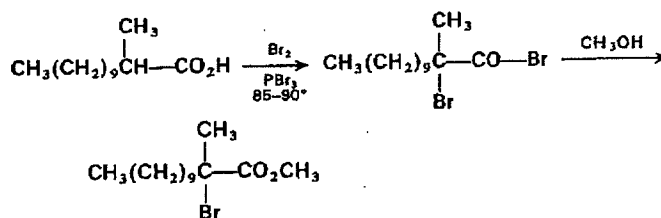March 12, 2008

Date
685927.1

Raghavan Rajagopalan, Ph.D.

# MODERN SYNTHETIC
# REACTIONS

## Second edition

Herbert O. House
*Georgia Institute of Technology*

BENJAMIN, INC.
Park, California

(Ref. 79)

the rate-limiting enolization of an acid chloride or acid anhydride intermediate, followed by reaction of the enol with the halogen. However, recent studies of these reactions[73b,c] are not consistent with such a reaction mechanism and suggest that some other reaction intermediate (possibly a ketene) is involved. A more satisfactory procedure for the bromination of acid chlorides involves their reaction with N-bromosuccinimide, as indicated below. This method does not appear to involve a free-radical chain reaction; however, which reaction path is being followed remains to be established.
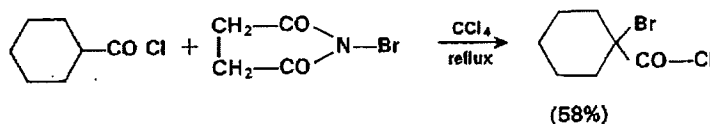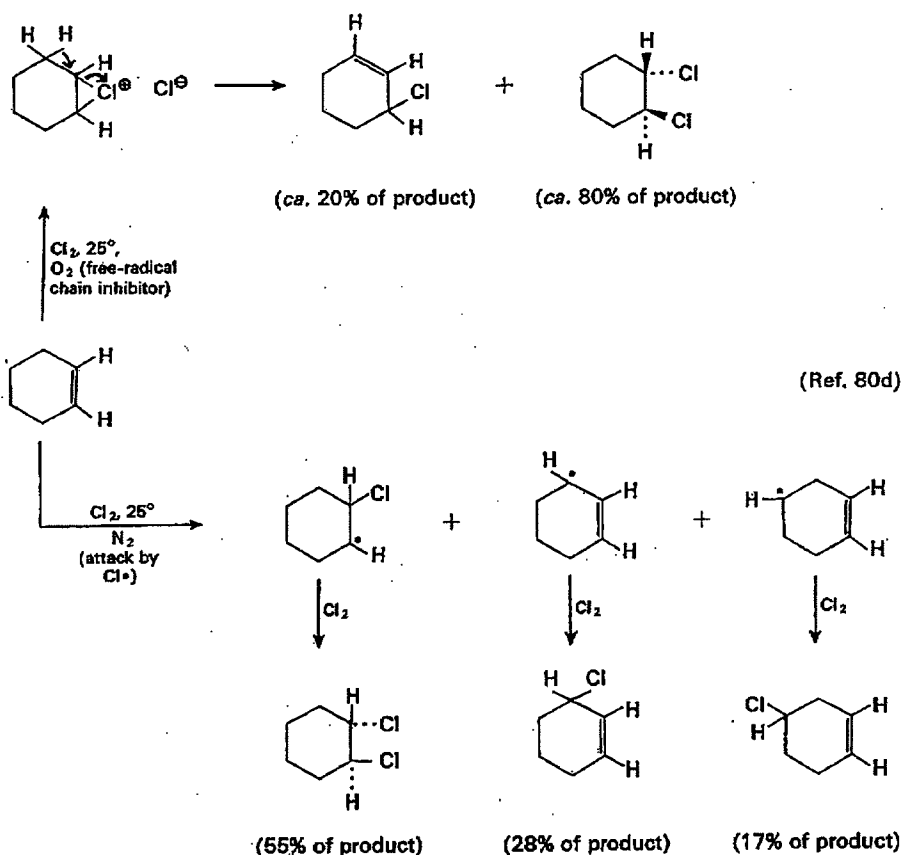


(58%)        (Ref. 75d)

## SUBSTITUTION OF HALOGEN AT BENZYLIC AND ALLYLIC CARBON-HYDROGEN BONDS

Unlike the previously discussed halogenations, the majority of reactions that substitute bromine or chlorine at an allylic or benzylic position appear to be free-radical chain processes.[80] However, as the following example illustrates, either ionic or free-radical pathways may lead to addition products and allylic halides. The substitution reactions are frequently run at elevated temperatures or are promoted by free-radical initiators such as light, a peroxide [usually dibenzoyl peroxide, $C_6H_5CO$—$OO$—$COC_6H_5$, or di-$t$-butyl peroxide, $(CH_3)_3C$—$OO$—$C(CH_3)_3$], or an azo compound [usually azobisisobutyronitrile, $(CH_3)_2C(CN)$—$N$=$N$—$C(CN)CH_3)_2$]. The commonly employed chlorinating agents are molecular chlorine, sulfuryl chloride $(SO_2Cl_2)$,[81b] trichloromethanesulfonyl chloride, and

79. C. F. Allen and M. J. Kalm, *Org. Syn.*, Coll. Vol. **4**, 608 (1963).
80. (a) C. Walling, *Free Radicals in Solution*, Wiley, New York, 1957, pp. 347–396; W. A. Pryor, *Free Radicals*, McGraw-Hill, New York, 1966, pp. 179–213; M. L. Poutsma in E. S. Huyser, ed., *Methods in Free Radical Chemistry*, Vol. 1, Marcel Dekker, New York, 1969, pp. 79–193; W. A. Thaler in E. S. Huyser, ed., *Methods in Free Radical Chemistry*, Vol. 2, Marcel Dekker, New York, 1969, pp. 121–227. (b) E. S. Huyser, *Synthesis*, 2, 7 (1970). (c) J. M. Tedder, *Quart. Rev.*, **14**, 336 (1960). (d) M. L. Poutsma, *J. Am. Chem. Soc.*, **87**, 2161, 2172 (1965).
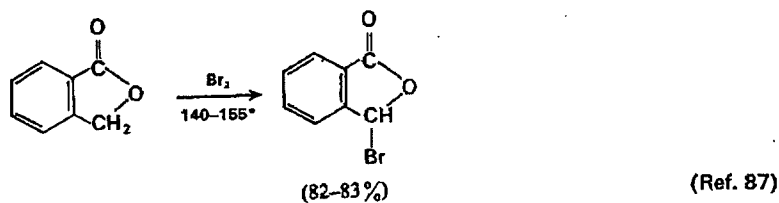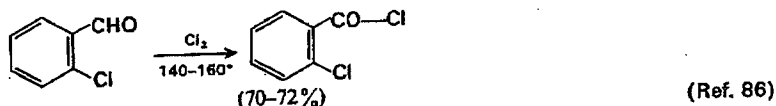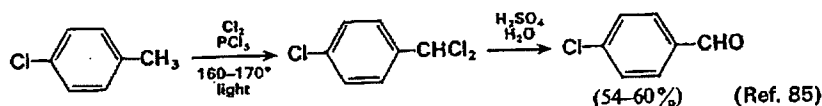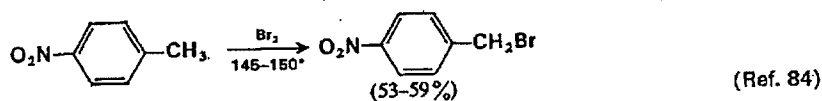81. (a) The preparation of this reagent is described by H. M. Teeter and E. W. Bell, *Org. Syn.*, Coll. Vol. **4**, 125 (1963), and M. J. Mintz and C. Walling, *ibid.*, **49**, 9 (1969). (b) K. H. Lee, *Tetrahedron*, **25**, 4357, 4363 (1969); 26, 2041 (1970).
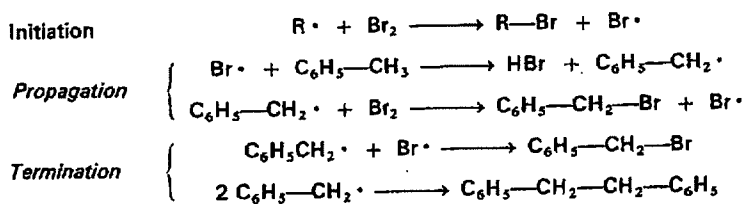
*(ca.* 20% of product)    *(ca.* 80% of product)

Cl₂, 25°,
O₂ (free-radical
chain inhibitor)

(Ref. 80d)

Cl₂, 25°
N₂
(attack by
Cl•)

·(55% of product)    (28% of product)    (17% of product)

*t*-butyl hypochlorite,[81a,82] whereas either molecular bromine or N-bromosuccini-
mide[3,83] is normally used for bromination.

In the absence of other reactive functional groups, halogenation at a benzylic
position is most readily effected with molecular bromine or chlorine, as in the
accompanying examples.

82. (a) C. Walling and B. B. Jacknow, *J. Am. Chem. Soc.,* **82**, 6108, 6113 (1960).   (b) C.
Walling and W. Thaler, *ibid.,* **83**, 3877 (1961).   (c) C. Walling and P. S. Fredricks, *ibid.,*
**84**, 3326 (1962).   (d) C. Walling and P. J. Wagner, *ibid.,* **86**, 3368 (1964).   (e) C. Walling
and M. J. Mintz, *ibid.,* **89**, 1515 (1967).   (f) C. Walling and J. A. McGuinness, *ibid.,* **91**, 2053
(1969).   (g) D. J. Carlsson and K. U. Ingold, *ibid.,* **89**, 4885, 4891 (1967).   (h) C. Walling
and V. P. Kurkov, *ibid.,* **89**, 4895 (1967).
83. (a) C. Djerassi, *Chem. Rev.,* **43**, 271 (1948);  L. Horner and E. H. Winkelmann, in
W. Foerst, ed., *Newer Methods of Preparative Organic Chemistry,* Vol. **3**, Academic Press,
New York, 1964, pp. 151–198.   (b) T. D. Waugh, *N-Bromosuccinimide: Its Reactions and*
*Use,* Arapahoe Chemicals, Inc., Boulder, Colorado, 1951.   (c) H. J. Dauben and L. L.
McCoy, *J. Am. Chem. Soc.,* **81**, 4863, 5404 (1959).   (d) For the preparation of N-iodo-
succinimide, see W. R. Benson, E. T. McBee, and L. Rand, *Org. Syn.,* **42**, 73 (1962).   (e) For
a review of the use of N-halo amides for oxidation reactions, see R. Filler, *Chem. Rev.,* **63**,
21 (1963) and Ref. 23d.

$O_2N$—⬡—$CH_3$ $\xrightarrow[145-150°]{Br_2}$ $O_2N$—⬡—$CH_2Br$

(53–59%)    (Ref. 84)

$Cl$—⬡—$CH_3$ $\xrightarrow[\substack{160-170° \\ light}]{\substack{Cl_2 \\ PCl_3}}$ $Cl$—⬡—$CHCl_2$ $\xrightarrow[H_2O]{H_2SO_4}$ $Cl$—⬡—$CHO$

(54–60%)    (Ref. 85)

⬡ with CHO and Cl $\xrightarrow[140-160°]{Cl_2}$ ⬡ with CO—Cl and Cl

(70–72%)    (Ref. 86)

⬡ with C=O, O, CH_2 $\xrightarrow[140-155°]{Br_2}$ ⬡ with C=O, O, CH, Br

(82–83%)    (Ref. 87)

The course of these reactions is illustrated in the scheme below. The hydrogen-abstraction step in the reactions constituting the propagation stage is retarded by the presence of electron-withdrawing substituents.[80,81b] As a result, each successive

**Initiation**    $R\cdot + Br_2 \longrightarrow R$—$Br + Br\cdot$

**Propagation** $\begin{cases} Br\cdot + C_6H_5$—$CH_3 \longrightarrow HBr + C_6H_5$—$CH_2\cdot \\ C_6H_5$—$CH_2\cdot + Br_2 \longrightarrow C_6H_5$—$CH_2$—$Br + Br\cdot \end{cases}$

**Termination** $\begin{cases} C_6H_5CH_2\cdot + Br\cdot \longrightarrow C_6H_5$—$CH_2$—$Br \\ 2\, C_6H_5$—$CH_2\cdot \longrightarrow C_6H_5$—$CH_2$—$CH_2$—$C_6H_5 \end{cases}$

halogen atom substituted at a benzylic position makes more difficult the abstraction of other hydrogen atoms at that position, a situation that facilitates the stepwise substitution of halogen (e.g., [61] and [62]). This same retarding effect by electron-withdrawing substituents is found in halogenations with sulfuryl chloride, t-butyl hypochlorite, or N-bromosuccinimide.

84. G. H. Coleman and G. E. Honeywell, *Org. Syn.,* Coll. Vol. 2, 443 (1943).
85. W. L. McEwen, *Org. Syn.,* Coll. Vol. 2, 133 (1943).
86. H. T. Clarke and E. R. Taylor, *Org. Syn.,* Coll. Vol. 1, 155 (1944).
87. R. L. Shriner and F. J. Wolf, *Org. Syn.,* Coll. Vol. 3, 737 (1955).

(Ref. 88)



(Ref. 89)



(Ref. 90)
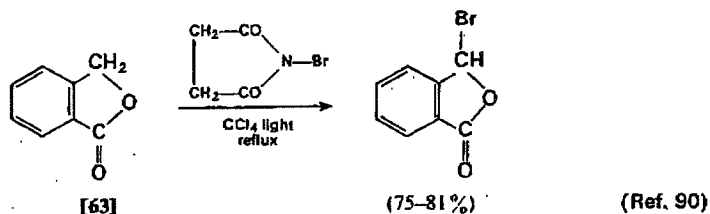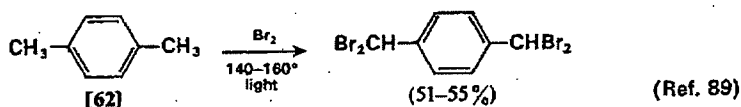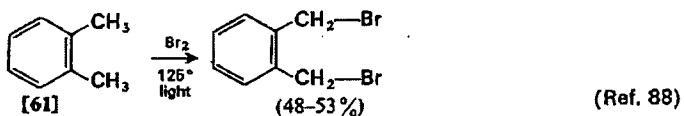
Benzylic bromination with N-bromosuccinimide is often a more convenient procedure (e.g., [63]) than bromination with molecular bromine and is definitely the method of choice in the case of a reactive aromatic nucleus (e.g., [64]) or in the presence of another functional group that can react with bromine (e.g., [65])



(Ref. 91a)



(ca. 100%)

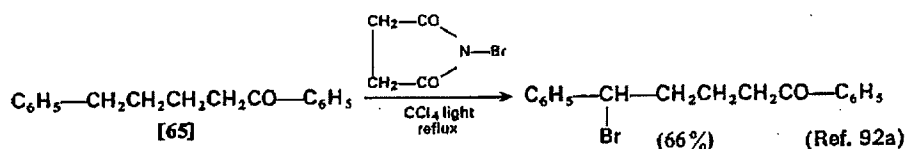(Ref. 91c)
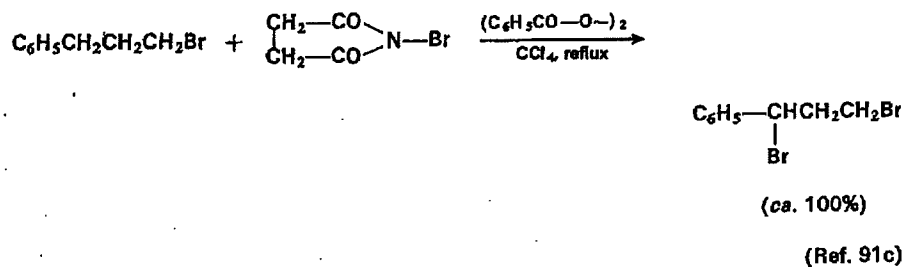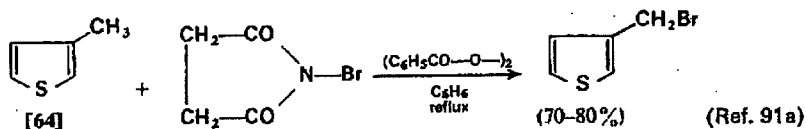
88. E. F. M. Stephenson, *Org. Syn.*, Coll. Vol. 4, 984 (1963).
89. J. M. Snell and A. Weissberger, *Org. Syn.*, Coll. Vol. 3, 788 (1955).
90. I. A. Koten and R. J. Sauer, *Org. Syn.*, 42, 26 (1962).
91. (a) E. Campaigne and B. F. Tullar, *Org. Syn.*, Coll. Vol. 4, 921 (1963).  (b) A. Kalir, *ibid.*, 46, 81 (1966).  (c) T. F. Corbin, R. C. Hahn, and H. Shechter, *ibid.*, 44, 30 (1964).

$$C_6H_5-CH_2CH_2CH_2CH_2CO-C_6H_5 \quad \xrightarrow[\text{reflux}]{\text{CCl}_4\text{ light}} \quad C_6H_5-CH-CH_2CH_2CH_2CO-C_6H_5$$

[65]                                                          Br  (66%)          (Ref. 92a)

or hydrogen bromide. Allylic and benzylic brominations with N-bromosuccinimide had originally been suggested[80,83] to proceed by a free-radical chain mechanism, with hydrogen abstraction by the succinimide radical. However, recent studies[80,93] indicate that a bromine radical is the hydrogen-abstracting agent in benzylic bromination and strongly suggest a similar mechanism for allylic bromination. According to this scheme, the bromine that is produced slowly by reaction of the N-bromosuccinimide with hydrogen bromide, as indicated below, enters into the previously illustrated free-radical chain reaction. If N-bromosuccinimide as used in polar acid solvents such as acetic acid or aqueous sulfuric acid, this acid-catalyzed process predominates. In such reaction media, aromatic hydrocarbons are brominated at the aryl nucleus rather than at benzylic positions.[92b,c]
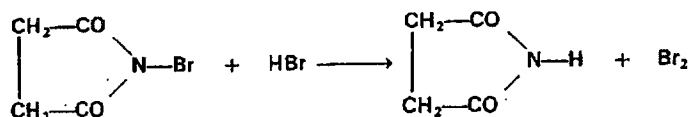
The halogenation of sterically hindered benzyl derivatives may lead to the rearrangement and/or elimination reaction of the radical intermediates, as illustrated in the following equation.
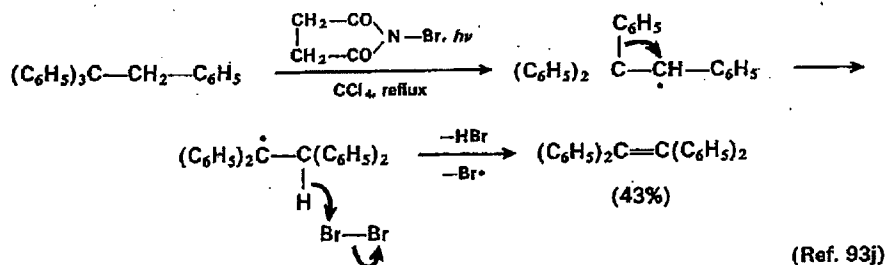
92. (a) R. L. Huang and P. Williams, *J. Chem. Soc.*, 2637 (1958). (b) F. Dewhurst and P. K. J. Shah, *J. Chem. Soc.*, C, 1737 (1970). (c) For the use of iodine and peracetic acid in acetic acid to iodinate the nucleus of aromatic hydrocarbons, see Y. Ogata and I. Urasaki, *ibid.*, C, 1689 (1970).

93. (a) R. E. Pearson and J. C. Martin, *J. Am. Chem. Soc.*, 85, 354, 3142 (1963); J. H. Incremona and J. C. Martin, *ibid.*, 92, 627 (1970). (b) B. P. McGrath and J. M. Tedder, *Proc. Chem. Soc.*, 80 (1961). (c) P. S. Skell, D. L. Tuleen, and P. D. Readio, *J. Am. Chem. Soc.*, 85, 2850 (1963). (d) E. Hedaya, R. L. Hinman, V. Schomaker, S. Theodoropulos, and L. M. Kyle, *ibid.*, 89, 4875 (1967). (e) C. Walling, A. L. Rieger, and D. D. Tanner, *ibid.*, 85, 3129 (1963). (f) G. A. Russell and K. M. Desmond, *ibid.*, 85, 3139 (1963). (g) T. Koenig and W. Brewer, *ibid.*, 86, 2728 (1964).    (h) R. L. Huang and K. H. Lee, *J. Chem.*

Bromine radicals generated photochemically[93m] or by radiolysis[91] will attack relatively unactivated C—H bonds. As the accompanying examples illustrate, tertiary alkyl radicals generated in this way in an excess of a hydrocarbon solvent



(Ref. 93m)



(70% of mixture)    (30% of mixture)    (Ref. 93m)
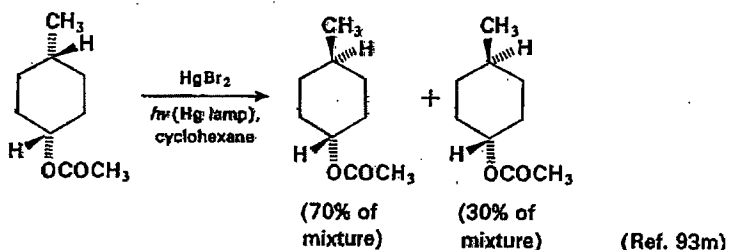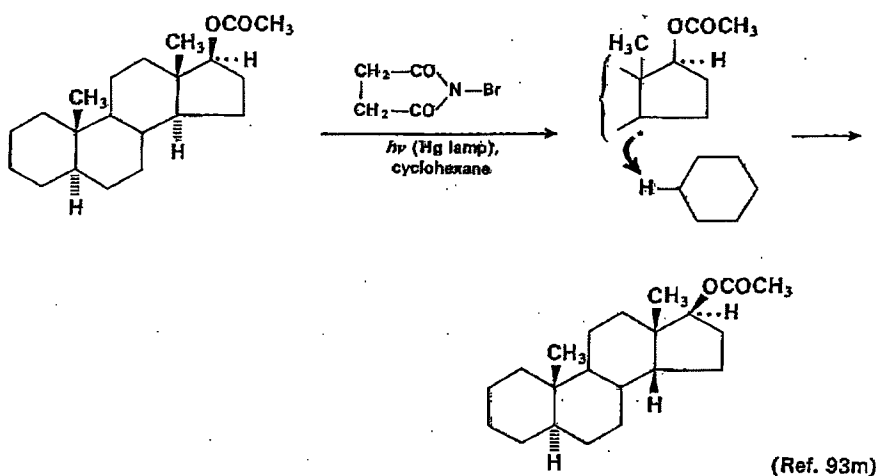
tend to abstract a hydrogen atom from the solvent. The overall reaction sequence serves to epimerize asymmetric centers which can be attacked by a bromine atom to form a relatively stable tertiary alkyl radical.

It has been shown that, at low concentration levels and in the absence of hydrogen bromide, bromine reacts with cyclohexene to form the allylic bromide [66] rather than the addition product, a 1,2-dibromide.[93b] Failure to observe addition of bromine to the double bond under these circumstances has been attributed to the reversibility of the first step in the addition reaction (by either a radical or an

*Soc.*, C, 932, 935 (1966) ; T. P. Low and K. H. Lee, *ibid.*, B, 535 (1970). (i) S. S. Friedrich, E. C. Friedrich, L. J. Andrews, and R. M. Keefer, *J. Org. Chem.*, 34, 900 (1969) ; I. Horman, S. S. Friedrich, R. M. Keefer, and L. J. Andrews, *ibid.*, 34, 905 (1969). (j) H. Meislich, J. Costanza, and J. Strelitz, *ibid.*, 33, 3221 (1968). (k) J. M. Landesberg and M. Siegel, *ibid.*, 35, 1674 (1970). (l) D. H. Martin and F. Williams, *J. Am. Chem. Soc.*, 92, 769 (1970). (m) M. Gorodetsky, D. Kogan, and Y. Mazur, *ibid.*, 92, 1094 (1970) ; M. Gorodetsky and Y. Mazur, *ibid.*, 90, 6540 (1968).

**[66]**
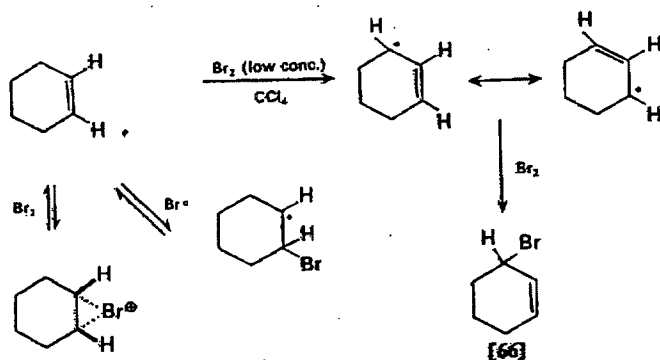
ionic mechanism). Since the concentrations of both bromine and hydrogen bromide (or bromide ion) are kept very low, little of the radical or ionic intermediate leading to addition is trapped to form the 1,2-dibromide. Under special circumstances, N-bromosuccinimide and its derivatives may be induced to undergo either free-radical or ionic additions to olefins. Thus, the reaction of N-bromosuccinimide with 3-sulfolene, an olefin with an electron-withdrawing sulfone function deactivating the allylic C—H bonds, yields an adduct which appears to



(8%)    (Ref. 93k)
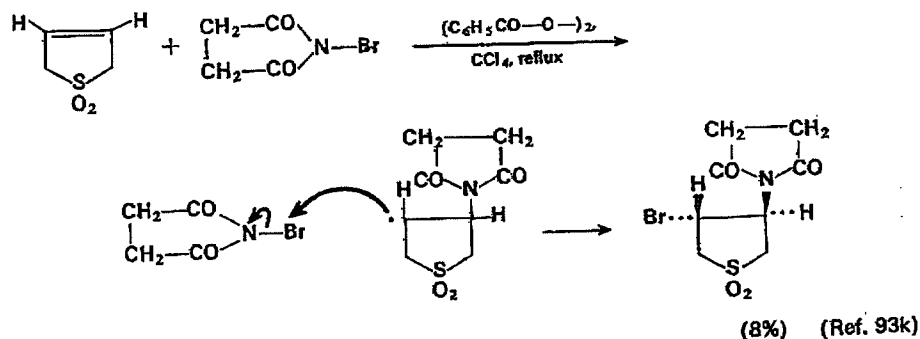
arise from a radical addition reaction. The reaction of 1,1-diphenylpropene with N-bromotetrafluorosuccinimide yields a similar adduct by a process which appears to be ionic. The latter reaction path is also capable of yielding allylic halides if the succinimide anion abstracts a proton from the bromonium ion.[93a]



(60%)    (Ref. 93a)
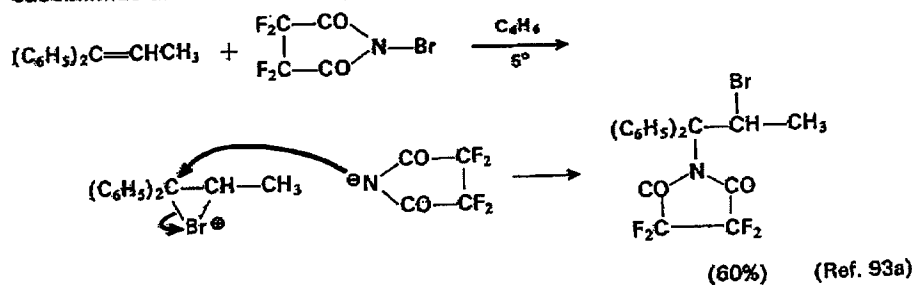
The following equations illustrate the use of N-bromosuccinimide for allylic bromination. Since a free allylic carbon radical (e.g., [67] from olefin [68]) is



$$CH_3(CH_2)_4CH_2—CH=CH_2 \xrightarrow[\substack{(C_6H_5CO—O—)_2 \\ CCl_4, \text{ reflux}}]{} CH_3(CH_2)_4\dot{C}H—CH=CH_2 \leftrightarrow CH_3(CH_2)_4CH=CH—\dot{C}H_2$$

[68]                                              [67a]                          [67b]

$$Br_2 \text{ or } \begin{array}{c} CH_2—CO \\ | \quad\quad N—Br \\ CH_2—CO \end{array}$$



$$CH_3(CH_2)_4—\underset{\underset{Br}{|}}{C}H—CH=CH_2 + CH_3(CH_2)_4\underset{H}{\overset{H}{\diagdown}}C=C\overset{CH_2Br}{\underset{H}{\diagup}} \quad + \quad \underset{H}{\overset{CH_3(CH_2)_4}{\diagdown}}C=C\overset{CH_2Br}{\underset{H}{\diagup}}$$

(17%)                    (44%)                          (39%)

(Ref. 94)

$$(CH_3)_2CH—CH=CH—CO_2C_2H_5 \xrightarrow[CCl_4, \text{ reflux}]{} (CH_3)_2\underset{\underset{Br}{|}}{C}—CH=CH—CO_2C_2H_5$$

(81%)          (Ref. 95a)

$$\underset{CH_3}{\overset{H}{\diagdown}}C=C\overset{CO_2CH_3}{\underset{CH_3}{\diagup}} \xrightarrow[\substack{(C_6H_5—CO—O—)_2, \\ CCl_4 \text{ reflux}}]{}$$

$$\underset{BrCH_2}{\overset{H}{\diagdown}}C=C\overset{CO_2CH_3}{\underset{CH_3}{\diagup}} \quad \underset{CH_3}{\overset{H}{\diagdown}}C=C\overset{CO_2CH_3}{\underset{CH_2Br}{\diagup}}$$

(45%)                    (20%)

(Ref. 95c, d)
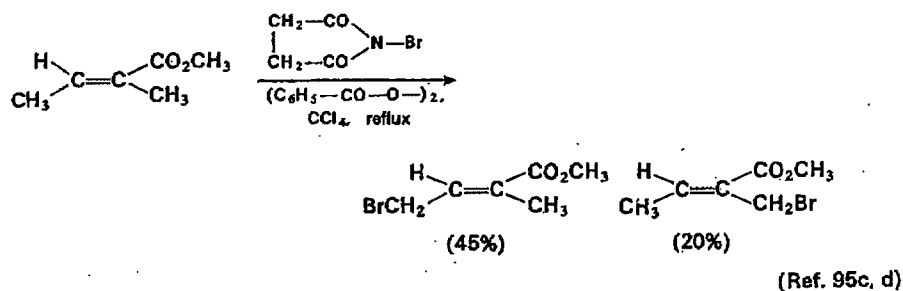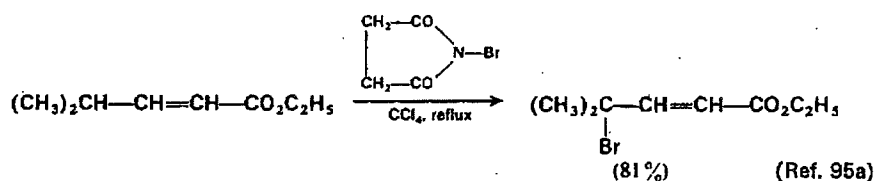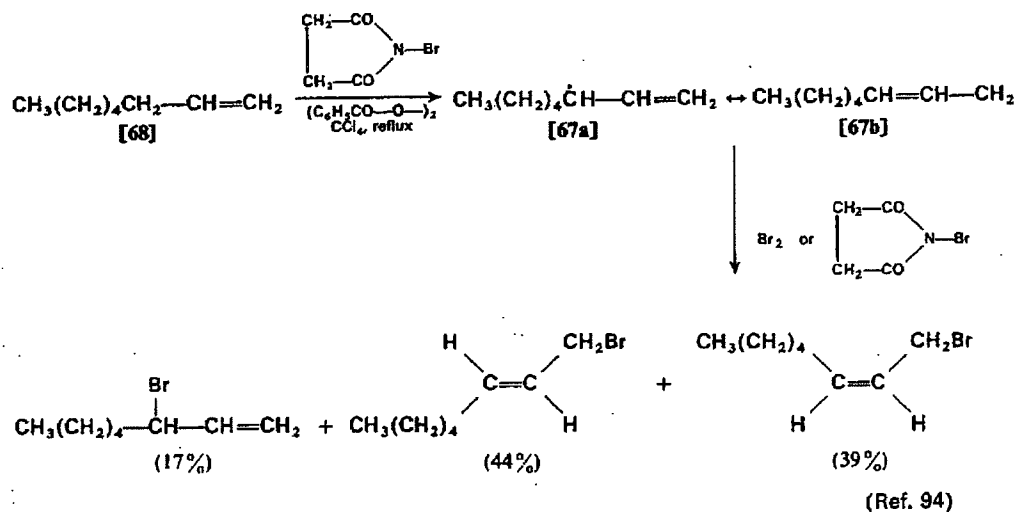
94. L. Bateman and J. I. Cunneen, *J. Chem. Soc.*, 941 (1950).
95. (a) H. J. Dauben, Jr., and L. L. McCoy, *J. Org. Chem.*, **24**, 1577 (1959). (b) I. Ahmad, R. N. Gedye, and A. Nechvatal, *J. Chem. Soc.*, C, **185** (1968). (c) A. Löffler, R. J. Pratt, H. P. Rüesch, and A. S. Dreiding, *Helv. Chim. Acta*, **53**, 383 (1970). (d) A. Löffler, F. Norris, W. Taub, K. L. Svanholt, and A. S. Dreiding, *ibid.*, **53**, 403 (1970).

presumably[80,83] an intermediate in these reactions no matter what the actual nature of the brominating agent, a mixture of allylic halides as products is to be expected.[80] The reported formation of only a single structural isomer from bromination of the olefin [69] therefore seems most curious; on the other hand, the absence of

$$CH_3(CH_2)_3-CH=CH-CH_3 \xrightarrow[\substack{(C_6H_5CO-O-)_2 \\ CCl_4, \text{ reflux}}]{} CH_3(CH_2)_2-CH-CH=CH-CH_3$$

[69]                                                    Br
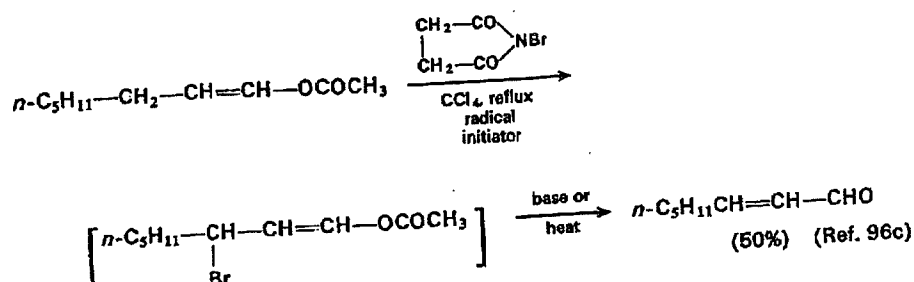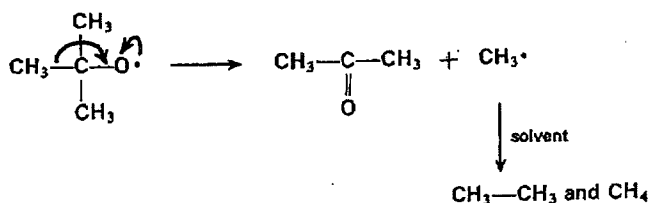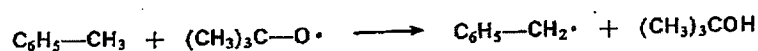
(58–64%)    (Ref. 92a, b)
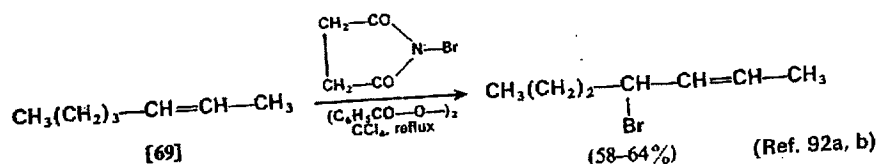
terminal unsaturation or of a bromomethyl group in the product is in agreement with the generalization[83] that a hydrogen atom is abstracted from secondary allylic positions more readily than from primary allylic positions. A similar situation has been observed in the bromination of $\alpha,\beta$-unsaturated esters. As the accompanying examples indicate, products with the bromine at the alpha carbon (adjacent to the deactivating carboalkoxyl group) have not been observed.[95] It should be noted that this result with $\alpha,\beta$-unsaturated esters could also arise from an ionic bromination involving a dienol intermediate from the starting ester.

Application of this allylic bromination to enol acetates yields intermediates which can be converted to $\alpha,\beta$-unsaturated aldehydes. Cyclic acetals are also

$$n\text{-}C_5H_{11}-CH_2-CH=CH-OCOCH_3 \xrightarrow[\substack{CCl_4 \text{ reflux} \\ \text{radical} \\ \text{initiator}}]{}$$

$$\left[ n\text{-}C_5H_{11}-\underset{\underset{Br}{|}}{CH}-CH=CH-OCOCH_3 \right] \xrightarrow[\text{heat}]{\text{base or}} n\text{-}C_5H_{11}CH=CH-CHO$$

(50%)  (Ref. 96c)

attacked by bromine radicals to yield the fragmentation products indicated. In certain cases where olefins have been brominated in reaction media containing some water, the initially formed allylic halides have been hydrolyzed to alcohols which were oxidized to $\alpha,\beta$-unsaturated ketones.[96e]

96. (a) F. L. Greenwood, M. D. Kellert, and J. Sedlak, Org. Syn., Coll. Vol. 4, 108 (1963). (b) F. L. Greenwood and M. D. Kellert, J. Am. Chem. Soc., 75, 4842 (1953). (c) J. J. Riehl and F. Jung, Tetrahedron Letters, No. 37, 3139 (1969). (d) J. D. Prugh and W. C. McCarthy, ibid., No. 13, 1351 (1966). It should be noted that the alpha C—H bonds of ethers are cleaved by aqueous bromine in a process which appears to involve ionic intermediates. N. C. Deno and N. H. Potter, J. Am. Chem. Soc., 89, 3550, 3555 (1967). (e) B. W. Finucane and J. B. Thompson, Chem. Commun., No. 20, 1220 (1969).

$$C_6H_5—CH_3 + (CH_3)_3C—O \cdot \longrightarrow C_6H_5—CH_2 \cdot + (CH_3)_3COH$$

$$C_6H_5CH_2 \cdot \curvearrowright Cl—O—C(CH_3)_3 \longrightarrow C_6H_5CH_2Cl + (CH_3)_3C—O \cdot$$

$$CH_3—\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle CH_3}{|}}{C}}—O \cdot \longrightarrow CH_3—\overset{\overset{\displaystyle }{\|}}{\underset{\underset{\displaystyle O}{}}{C}}—CH_3 + CH_3 \cdot$$

$\downarrow$ solvent

$$CH_3—CH_3 \text{ and } CH_4$$

Acetylenes and allenes undergo a chlorination reaction similar to that observed with olefins. However, the intermediate propargylic radical [71] appears to react with chlorine or *t*-butyl hypochlorite to form acetylenic products containing little if any of the isomeric allenes.[98] *t*-Butyl hypochlorite has also been used to chlorinate

$$C_6H_5—CH_2C\equiv CH \xrightarrow[h\nu \text{ (tungsten lamp) } 0°]{(CH_3)_3C—O—Cl}$$

$$C_6H_5—\underset{\cdot}{CH}—C\equiv C—H$$

$\updownarrow$

$$C_6H_5—CH=C=\underset{\cdot}{CH}$$
$$[71]$$

$$\xrightarrow{(CH_3)_3C—O—Cl} C_6H_5\underset{\underset{\displaystyle Cl}{|}}{CH}—C\equiv CH$$

(Ref. 98a)
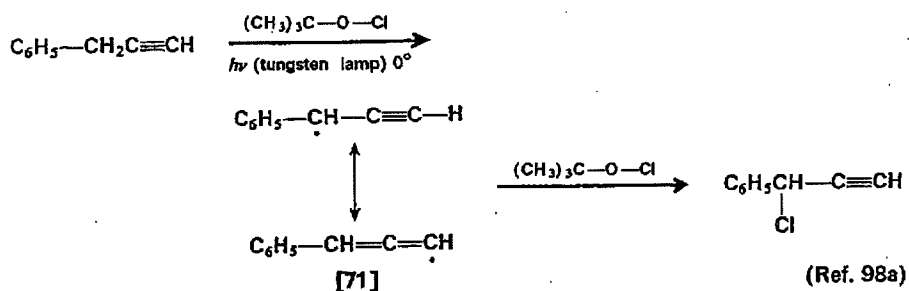
ethers, alcohols, aldehydes, ketones, and amines. The following equations provide examples of these uses.



(48% of product)    (52% of product)    (Ref. 82e)



(Ref. 82e)
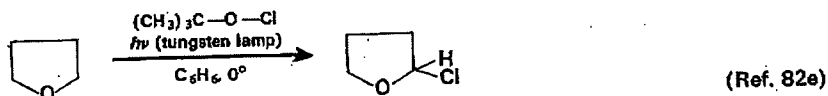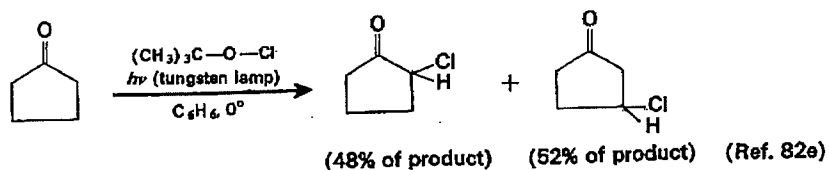
98. (a) M. C. Caserio and R. E. Pratt, *Tetrahedron Letters*, No. 1, 91 (1967). (b) M. L. Poutsma and J. L. Kartch, *Tetrahedron*, 22, 2167 (1966). (c) R. M. Fantazier and M. L. Poutsma, *J. Am. Chem. Soc.*, 90, 5490 (1968). (d) L. R. Byrd and M. C. Caserio, *Ibid.*, 92, 5422 (1970). (e) J. K. Kochi and P. J. Krusic, *Ibid.*, 92, 4110 (1970).

$$C_6H_5-CH-CH_3 + (CH_3)_3C-O-Cl \xrightarrow[5-25°]{C_6H_6} C_6H_5CH-CH_3 \xrightarrow[C_6H_6, \text{reflux}]{CH_3ONa, CH_3OH}$$
$$\underset{NH_2}{\mid} \qquad (2 \text{ equiv.}) \qquad \underset{NCl_2}{\mid}$$

$$\overset{NH}{\underset{OCH_3}{\overset{\triangle}{C_6H_5-C-CH_2}}} \xrightarrow[\substack{(CH_3)_2CHOH, \\ \text{reflux}}]{HCl, H_2O} C_6H_5-CO-CH_2\overset{\oplus}{N}H_3\overset{\ominus}{Cl}$$

$$(55-72\%) \qquad (\text{Ref. 99})$$
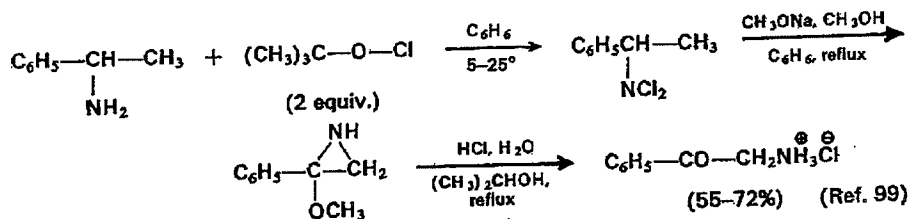
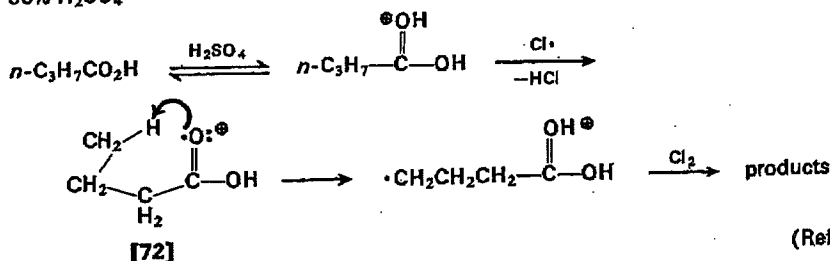Free-radical halogenations of saturated compounds have been studied extensively[30] to establish the reactivity of various C—H bonds to the hydrogen atom abstraction reaction. These studies have established the reactivity order, tertiary CH> secondary CH> primary CH, and have also established the deactivating effect of nearby electron-withdrawing substituents.[30,82e,100] In spite of these selectivities in free-radical halogenation of alkanes, the halogenation of substrates without specially activated C—H bonds (e.g., benzyl or allyl CH bonds) frequently yields mixtures of halogenated products which are difficult to separate on a preparative scale. Notable exceptions are the previously discussed (see Chapter 7) *intra-molecular* abstractions of a hydrogen atom from an unactivated C—H bond by a radical which is generated and held near a particular C—H bond. As indicated in Chapter 7, these radicals have been generated from alcohols and lead tetraacetate, from alkyl hypohalites, from N-halo amides, or from protonated N-halo amines. The following equations compare the product compositions from the free-radical

$$CH_3CH_2CH_2CO_2H \xrightarrow[\text{solvent}]{\substack{Cl_2, 25° \\ h\nu}}$$

$$ClCH_2CH_2CH_2CO_2H + \underset{Cl}{\overset{\mid}{CH_3CHCH_2CO_2H}} + \underset{Cl}{\overset{\mid}{CH_3CH_2CHCO_2H}}$$
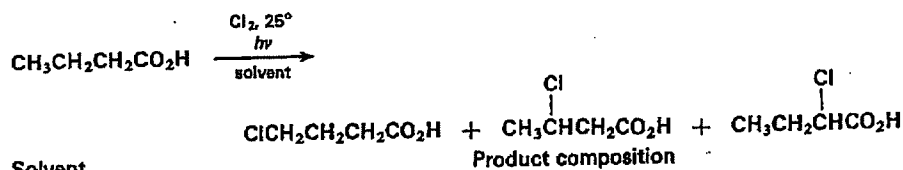
| Solvent | Product composition | | |
|---|---|---|---|
| CCl$_4$ | 42% | 53% | 5% |
| 90% H$_2$SO$_4$ | 79% | 21% | — |

$$n\text{-}C_3H_7CO_2H \underset{}{\overset{H_2SO_4}{\rightleftharpoons}} n\text{-}C_3H_7-\overset{\oplus OH}{\overset{\|}{C}}-OH \xrightarrow[-HCl]{Cl\cdot}$$

$$\underset{\overset{\mid}{H_2}}{\overset{CH_2}{\underset{CH_2}{\diagdown}}}\overset{H}{\underset{C}{\diagup}}\overset{\cdot \overset{\oplus}{O}:}{\underset{}{\diagdown}}{\overset{\|}{C}}-OH \longrightarrow \cdot CH_2CH_2CH_2-\overset{OH\oplus}{\overset{\|}{C}}-OH \xrightarrow{Cl_2} \text{products}$$

**[72]**

$$(\text{Ref. 100c})$$

99. (a) H. E. Baumgarten and J. M. Petersen, *Org. Syn.*, **41**, 82 (1961). (b) G. H. Alt and W. S. Knowles, *ibid.*, **45**, 16 (1965). (c) For a related procedure employing N,N-dichlorobenzenesulfonamide as the halogenating agent, see T. Taguchi, Y. Shimizu, and Y. Kawazoe, *Tetrahedron Letters*, No. **32**, 2853 (1970).
100. (a) D. S. Ashton and J. M. Tedder, *Chem. Commun.*, No. **14**, 785 (1968). (b) F. Minisci, R. Galli, and R. Bernardi, *ibid.*, No. **17**, 903 (1967); F. Minisci, R. Galli, A. Galli, and R. Bernardi, *Tetrahedron Letters*, No. **23**, 2207 (1967). (c) N. C. Deno, R. Fishbein, and J. C. Wyckoff, *J. Am. Chem. Soc.*, **92**, 5274 (1970).
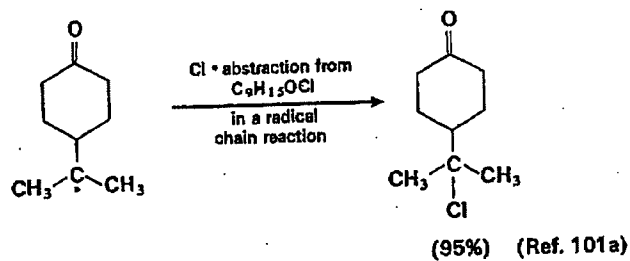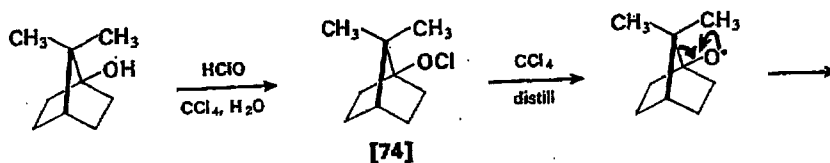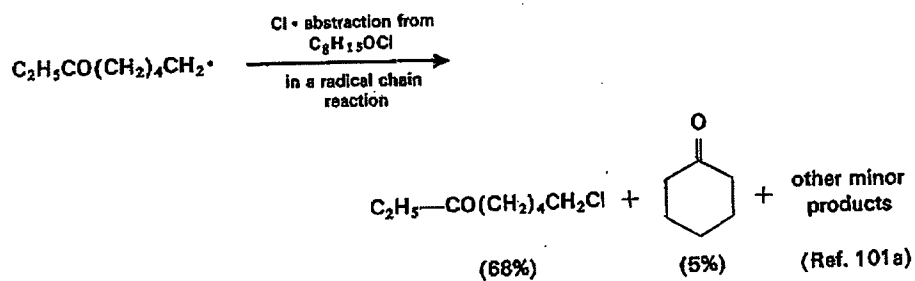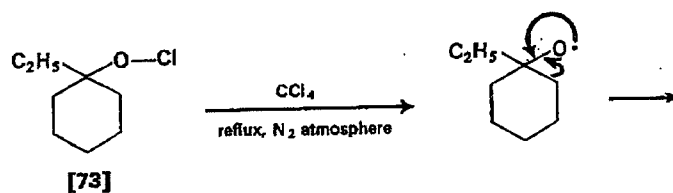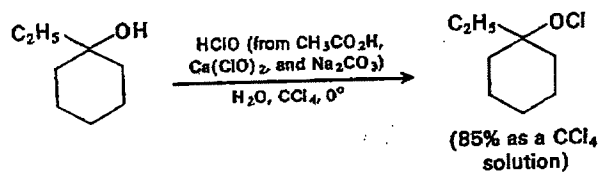
chlorination of butyric acid in a nonpolar solvent ($CCl_4$) and in 90% sulfuric acid. In both cases, very little substitution is observed alpha to the electron-withdrawing carbonyl group. The enhanced tendency for the reaction in 90% sulfuric acid to form the $\gamma$-chloro acid is suggested to result from an intramolecular hydrogen atom abstraction, as illustrated in structure [72].

This selective halogenation of an unactivated position may also be accomplished by the thermal or photochemical decomposition of t-alkyl hypochlorites,[101] as indicated in the following example. The fragmentation of the t-alkyl hypochlorites



(Ref. 101b)
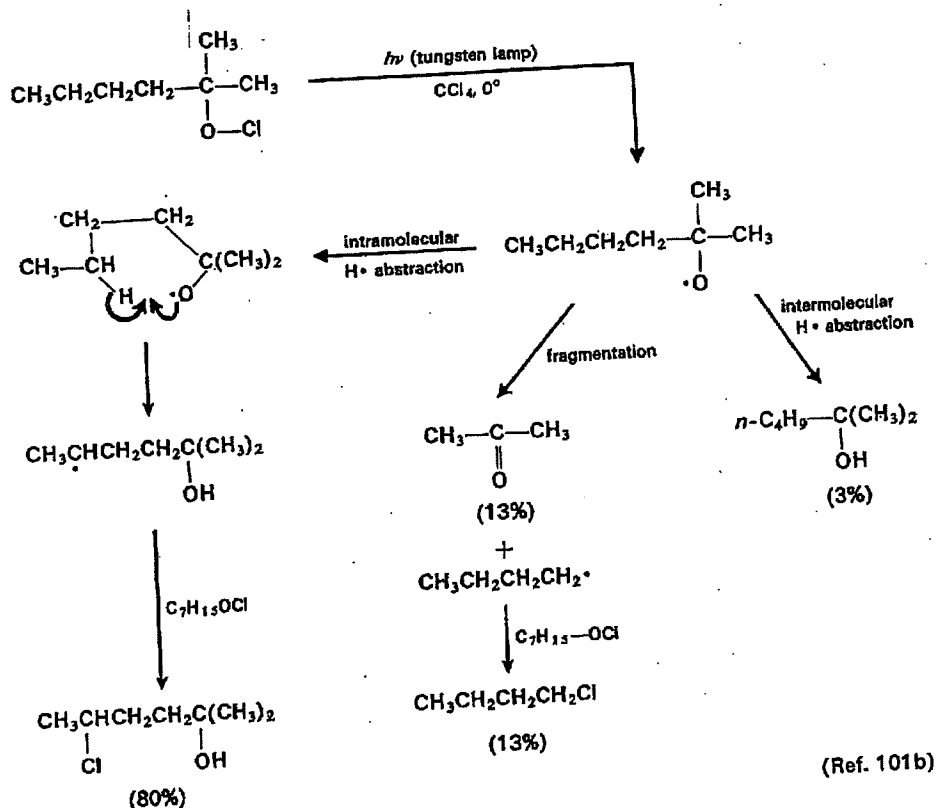
(e.g. [73] and [74]) can also serve as a useful procedure for the introduction of a halogen atom at a specific site with a free-radical chain process. As the accompanying equations indicate, the fragmentation of the alkoxyl radical intermediate occurs in such a way that a carbonyl compound and the most stable carbon radical are formed.

101. (a) F. D. Greene, M. L. Savitz, F. D. Osterholtz, H. H. Lau, W. N. Smith, and P. M. Zanet, J. Org. Chem., 28, 55 (1963). (b) C. Walling and A. Padwa, J. Am. Chem. Soc., 85, 1597 (1963). (c) M. Akhtar, P. Hunt, and P. B. Dewhurst, ibid., 87, 1807 (1965).

$C_2H_5$ OH

HClO (from $CH_3CO_2H$, Ca(ClO)$_2$, and Na$_2CO_3$)
$H_2O$, CCl$_4$, 0°

$C_2H_5$ OCl

(85% as a CCl$_4$ solution)

$C_2H_5$ O—Cl

CCl$_4$
reflux, N$_2$ atmosphere

$C_2H_5$ O•

[73]

$C_2H_5CO(CH_2)_4CH_2$•

Cl • abstraction from C$_8H_{15}$OCl

in a radical chain reaction

$C_2H_5$—CO(CH$_2$)$_4$CH$_2$Cl  +

(68%)

+

(5%)

+ other minor products

(Ref. 101a)

$CH_3$  $CH_3$
OH

HClO
CCl$_4$, $H_2O$

$CH_3$  $CH_3$
OCl

CCl$_4$
distill

$CH_3$  $CH_3$
O•

[74]

O

Cl • abstraction from C$_9H_{15}$OCl

in a radical chain reaction

O

$CH_3$ C $CH_3$

$CH_3$ C $CH_3$
Cl

(95%)   (Ref. 101a)

# Bioconjugate
## Techniques

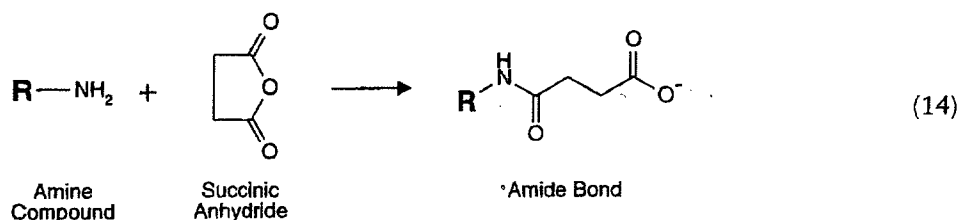**Greg T. Hermanson**
Pierce Chemical Company
Rockford, Illinois

**Academic Press**

San Diego    New York    Boston    London    Sydney    Tokyo    Toronto

$$R\text{—}NH_2 \;+\; \text{[Succinic Anhydride]} \;\longrightarrow\; \text{[Amide Bond product]} \qquad (14)$$

Amine            Succinic                    ·Amide Bond
Compound       Anhydride

N-terminals, the ε-amine of lysine side chains, cysteine sulfhydryl groups, the phenolate ion of tyrosine residues, and the imidazolyl ring of histidines. However, acylation of cysteine, tyrosine, and histidine side chains forms unstable complexes that are easily reversible to regenerate the original group. Only amine functional groups of proteins are stable to acylation with anhydride reagents, forming amide bonds (Fraenkel-Conrat, 1959; Smyth, 1967).

Another potential site of reactivity for anhydrides in protein molecules is modification of any attached carbohydrate chains. In addition to amino group modification in the polypeptide chain, glycoproteins may be modified at their polysaccharide hydroxyl groups to form esterified derivatives. Esterification of carbohydrates by acetic anhydride, especially cellulose, is a major industrial application for this compound. In aqueous solutions, however, esterification will be a minor product, since the oxygen of water is about as strong a nucleophile as the hydroxyls of sugar residues.

The major side reaction to the desired acylation product is hydrolysis of the anhydride. In aqueous solutions anhydrides may breakdown by the addition of one molecule of water to yield two unreactive carboxylate groups. The presence of an excess of the anhydride in the reaction medium usually is used to minimize the effects of competing hydrolysis.

Since both hydrolysis and acylation yield the release of carboxylic acid functional groups, the medium becomes acidic during the course of the reaction. This requires either the presence of a strongly buffered environment to maintain the pH or periodic monitoring and adjustment of the pH with base as the reaction progresses.
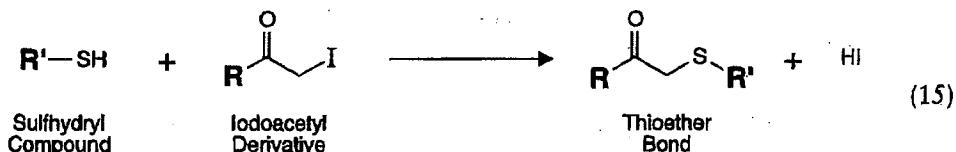
## 2. Thiol-Reactive Chemical Reactions

Reactive groups able to couple with sulfhydryl-containing molecules are perhaps the second most common functional groups present on cross-linking or modification reagents. Especially in the design of heterobifunctional cross-linkers, sulfhydryl-reactive groups frequently are present on one of the two ends. The other end of such cross-linkers is often an amine-reactive functional group that is coupled to a target molecule before the sulfhydryl-reactive end, due to the labile nature of amine acylation chemistries. The primary coupling chemical reactions for modification of sulfhydryls proceed by one of two routes: alkylation or disulfide interchange. Many of the reactive groups that undergo these reactions are stable enough in aqueous environments to allow a two-step conjugation strategy to be used (Chapter 5, Section 1). Once initiated, most of these reactions are rapid and occur in high yield to give stable thioether and disulfide bonds.

## 2.1. Haloacetyl and Alkyl Halide Derivatives

Three forms of activated halogen derivatives can be used to create sulfhydryl-reactive compounds; haloacetyl (see Chapter 1, Section 5.2), benzyl halides that react through a resonance activation process with the neighboring benzene ring, and alkyl halides that possess the halogen β to a nitrogen or sulfur atom, as in N- and S-mustards. In each of these compounds the halogen group is easily displaced by an attacking nucleophilic substance to form an alkylated derivative with loss of HX (where X is the halogen and the hydrogen comes from the nucleophile). Haloacetyl compounds and benzyl halides typically are iodo or bromo derivatives, whereas the halo-mustards mainly employ chloro and bromo forms (see Chapter 4, Section 10 for examples of homobifunctional reagents that employ reactive halogen groups).

Although the primary utility of active halogen compounds is to modify sulfhydryl groups in proteins or other molecules, the reaction is not totally specific. Iodoacetyl (and bromoacetyl) derivatives can react with a number of functional groups within proteins: the sulfhydryl group of cysteine, both imidazolyl side chain nitrogens of histidine, the thioether of methionine, and the primary ε-amine group of lysine residues and N-terminal α-amines (Gurd, 1967). The relative rate of reaction with each of these residues is generally dependent on the degree of ionization and thus the pH at which the modification is done. The exception to this rule is methioninyl thioethers, which react rapidly at nearly all pH values above 1.7 (Vithayathil and Richards, 1960). The only reaction resulting in one definitive product is that of the alkylation of cysteine sulfhydryls, giving the carboxymethylcysteinyl derivative (Cole *et al.*, 1958) (Reaction 15). Histidine groups may be modified at either nitrogen atom of its imidazolyl side chain, thus producing the possibility of either mono-substituted or di-substituted products (Crestfield *et al.*, 1963). With primary amine groups such as in the side chain of lysine residues, the products of the reaction are either the secondary amine monocarboxymethyllysine or the tertiary amine derivative dicarboxymethyllysine. Methionine thioether groups give the most complicated products, some of which rearrange or decompose unpredictably. The only stable carboxy derivative of methionine is where the terminal methyl group is lost to form carboxymethylhomocysteine, the same product as the reaction of iodoacetate with homocysteine. For a complete illustration of these reactions, see Chapter 4, Section 10.
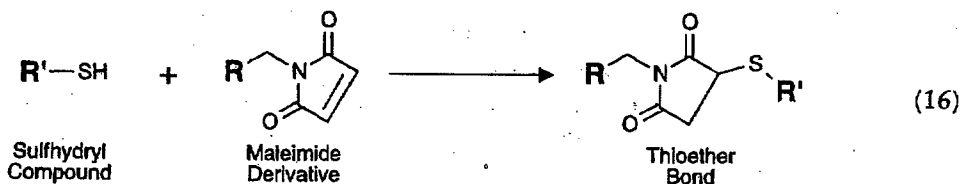
$$\text{R'}\text{—SH} \quad + \quad \underset{\underset{\text{Iodoacetyl}}{\text{Derivative}}}{\text{R}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—CH}_2\text{—I}} \quad \longrightarrow \quad \underset{\underset{\text{Thioether}}{\text{Bond}}}{\text{R}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—CH}_2\text{—S}\text{—R'}} \quad + \quad \text{HI} \qquad (15)$$

Sulfhydryl Compound

The relative reactivity of α-haloacetates toward protein functionalities is sulfhydryl > imidazolyl > thioether > amine. Among halo derivatives the relative reactivity is I > Br > Cl > F, with fluorine being almost unreactive. The α-haloacetamides have the same trend of relative reactivities, but will obviously not create a terminal carboxylate functional group.

Thus, iodoacetate has the highest reactivity toward sulfhydryl cysteine residues and may be directed specifically for —SH modification. If iodoacetate is present in limiting

quantities (relative to the number of sulfhydryl groups present) and at slightly alkaline pH, cysteine modification will be the exclusive reaction. The specificity of this modification has been used in the design of heterobifunctional cross-linking reagents, where one end of the cross-linker contains an iodoacetamide derivative and the other end contains a different functional group directed at another chemical target (see SIAB, Chapter 5, Section 5).

## 2.2. Maleimides

Maleic acid imides (maleimides) are derivatives of the reaction of maleic anhydride and ammonia. This functional group is a popular constituent of many heterobifunctional cross-linking agents (Chapter 5). The double bond of maleimides may undergo an alkylation reaction with sulfhydryl groups to form stable thioether bonds. Maleimide reactions are specific for sulfhydryl groups in the pH range 6.5–7.5 (Heitz *et al.*, 1968; Smyth *et al.*, 1964; Gorin *et al.*, 1966; Partis *et al.*, 1983). At pH 7, the reaction of the maleimide with sulfhydryls proceeds at a rate 1000 times greater than its reaction with amines. At higher pH values some cross-reactivity with amino groups takes place (Brewer and Riehm, 1967). One of the carbons adjacent to the maleimide double bond undergoes nucleophilic attack by the thiolate anion to generate the addition product (Reaction 16). When sufficient quantities of —SH groups are being alkylated, the reaction may be followed spectrophotometrically by the decrease in absorbance at 300 nm as the double bond reacts and disappears.



R'—SH   +   Maleimide Derivative   ⟶   Thioether Bond                    (16)

Sulfhydryl Compound          Maleimide Derivative              Thioether Bond

The maleimide group also may undergo hydrolysis to an open maleamic acid form that is unreactive toward sulfhydryls (Chapter 9, Section 5). Hydrolysis may occur after sulfhydryl coupling to the maleimide, as well. This ring-opening reaction typically happens faster the higher the pH becomes. Hydrolysis is also dependent on the type of chemical group next to the maleimide function. For instance, the cyclohexane ring of SMCC (Chapter 5, Section 1.3) provides increased stability to maleimide hydrolysis probably due to its steric effects and its lack of aromatic character. However, the adjacent phenyl ring of MBS allows much greater rates of hydrolysis to occur at the maleimide ring (Chapter 5, Section 1.4).

## 2.3. Aziridines

An aziridine functional group is a small ring system composed of one nitrogen and two carbon atoms. The highly hindered nature of this heterocyclic ring gives it strong reactivity toward nucleophiles. Sulfhydryls will react with aziridine-containing reagents in a ring-opening process, forming thioether bonds (Reaction 17). The simplest

# Organic Chemistry

## Second Edition

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and

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**Allyn and Bacon, Inc.**

*Boston*

# Pre

The reas
organize and ⁣
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we have tried
to offer him
language that
We have
to family, with
tion is realistic
each type of
organization i
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and free-radic
So that
carbonyl chen
nucleophilic a
In Chap
strange new la
spectra: mass
the workhorse
about NMR,
NMR. In foll
characteristics
classes.

The secondary amine, which is in equilibrium with its salt, can in turn attack the alkyl halide to form the salt of a tertiary amine:
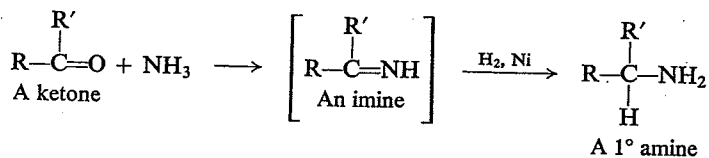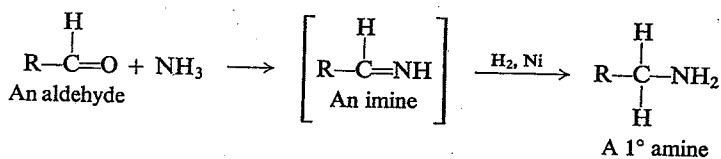
$$R_2NH + RX \longrightarrow R_3NH^+X^- \xrightarrow{\quad NH_3 \quad} R_3N$$

2° amine                                               3° amine
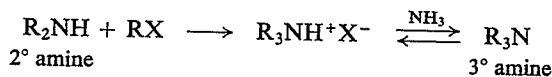
Finally, the tertiary amine can attack the alkyl halide to form a compound of the formula $R_4N^+X^-$, called a *quaternary ammonium salt* (discussed in Sec. 23.5):

$$R_3N + RX \longrightarrow R_4N^+X^-$$

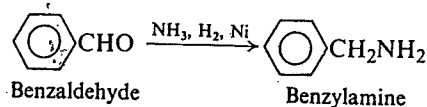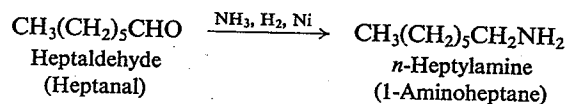3° amine          Quaternary ammonium salt
(4°)
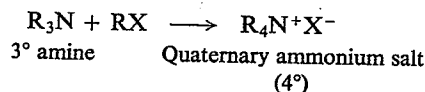
The presence of a large excess of ammonia lessens the importance of these last reactions and increases the yield of primary amine; under these conditions, a molecule of alkyl halide is more likely to encounter, and be attacked by, one of the numerous ammonia molecules rather than one of the relatively few amine molecules. At best, the yield of primary amine is always cut down by the formation of the higher classes of amines. Except in the special case of methylamine, the primary amine can be separated from these by-products by distillation.
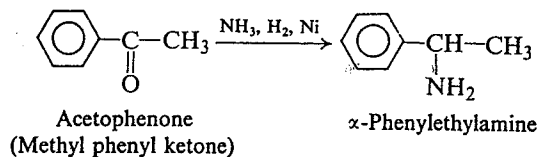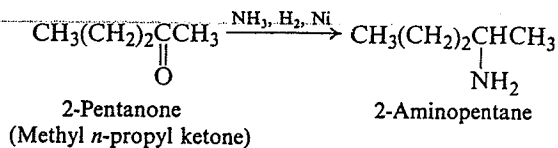
## 22.11 Reductive amination

Many aldehydes (RCHO) and ketones ($R_2CO$) are converted into amines by treatment with hydrogen and ammonia in the presence of a catalyst; this process is known as **reductive amination**. Although the mechanism is not clear, the reaction may involve hydrogenation of an intermediate compound (an *imine*, $RCH=NH$ or $R_2C=NH$) that contains a carbon–nitrogen double bond.

$$
\begin{array}{c}
\text{H} \\
\text{R—C=O + NH}_3 \\
\text{An aldehyde}
\end{array}
\longrightarrow
\left[
\begin{array}{c}
\text{H} \\
\text{R—C=NH} \\
\text{An imine}
\end{array}
\right]
\xrightarrow{\text{H}_2,\ \text{Ni}}
\begin{array}{c}
\text{H} \\
\text{R—C—NH}_2 \\
\text{H} \\
\text{A 1° amine}
\end{array}
$$

$$
\begin{array}{c}
\text{R}' \\
\text{R—C=O + NH}_3 \\
\text{A ketone}
\end{array}
\longrightarrow
\left[
\begin{array}{c}
\text{R}' \\
\text{R—C=NH} \\
\text{An imine}
\end{array}
\right]
\xrightarrow{\text{H}_2,\ \text{Ni}}
\begin{array}{c}
\text{R}' \\
\text{R—C—NH}_2 \\
\text{H} \\
\text{A 1° amine}
\end{array}
$$

Reductive amination has been used successfully with a wide variety of aldehydes and ketones, both aliphatic and aromatic. For example:

$$CH_3(CH_2)_5CHO \xrightarrow{\text{NH}_3,\ \text{H}_2,\ \text{Ni}} CH_3(CH_2)_5CH_2NH_2$$

Heptaldehyde                          *n*-Heptylamine
(Heptanal)                           (1-Aminoheptane)

Benzaldehyde $\xrightarrow{\text{NH}_3,\ \text{H}_2,\ \text{Ni}}$ Benzylamine

$$CH_3(CH_2)_2\overset{\underset{\|}{O}}{C}CH_3 \xrightarrow{NH_3,\ H_2,\ Ni} CH_3(CH_2)_2\overset{\underset{NH_2}{|}}{C}HCH_3$$

2-Pentanone          2-Aminopentane
(Methyl *n*-propyl ketone)

$$\underset{\text{Acetophenone}}{\bigcirc\hspace{-0.5em}-\overset{\underset{\|}{O}}{C}-CH_3} \xrightarrow{NH_3,\ H_2,\ Ni} \bigcirc\hspace{-0.5em}-\overset{\underset{NH_2}{|}}{C}H-CH_3$$

Acetophenone          α-Phenylethylamine
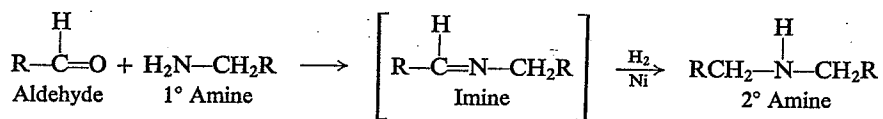(Methyl phenyl ketone)

Reductive amination of ketones yields amines containing a *sec*-alkyl group; such amines are difficult to obtain by ammonolysis because of the tendency for *sec*-alkyl halides to undergo elimination.    For example, cyclohexanone is converted into cyclohexylamine in good yield, whereas ammonolysis of bromocyclohexane yields only cyclohexene.
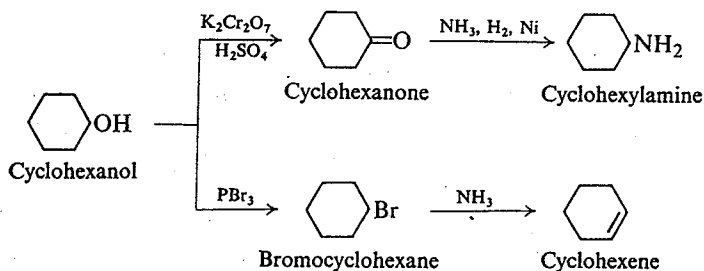


During reductive amination the aldehyde or ketone can react not only with ammonia but also with the primary amine that has already been formed, and thus yield a certain amount of secondary amine.    The tendency for the reaction to go

$$\underset{\text{Aldehyde}}{\overset{\underset{|}{H}}{R-C}=O} + \underset{\text{1° Amine}}{H_2N-CH_2R} \longrightarrow \left[\underset{\text{Imine}}{\overset{\underset{|}{H}}{R-C}=N-CH_2R}\right] \xrightarrow[Ni]{H_2} \underset{\text{2° Amine}}{RCH_2-\overset{\underset{|}{H}}{N}-CH_2R}$$

beyond the desired stage can be fairly well limited by the proportions of reactants employed and is seldom a serious handicap.

**Problem 22.5**  Using a different method in each case, show how the following amines could be prepared from *toluene* and any aliphatic reagents:

(a) $\bigcirc\hspace{-0.5em}-CH_2NH_2$          (c) $\bigcirc\hspace{-0.5em}-CH_2CH_2NH_2$

(b) $CH_3-\bigcirc\hspace{-0.5em}-\overset{\underset{NH_2}{|}}{C}HCH_3$          (d) $CH_3-\bigcirc\hspace{-0.5em}-NH_2$

(e) $\bigcirc\hspace{-0.5em}-NH_2$